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Identification, Expression, and Biochemical Characterization of *N*-Acetylgalactosamine-4-Sulfatase Mutations and Relationship with Clinical Phenotype in MPS-VI Patients

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Summary

Maroteaux-Lamy syndrome, or mucopolysaccharidosis type VI (MPS-VI), is a lysosomal storage disorder characterized by the defective degradation of dermatan sulfate due to the deficiency of *N*-acetylgalactosamine-4-sulfatase (4S). The clinical severity of MPS-VI ranges in a continuum from mildly affected to severely affected patients. Mutations in MPS-VI patient samples were identified by chemical cleavage and direct DNA sequencing of PCR products derived from patient cDNA. Five amino acid substitutions were identified (T92M, R95Q, Y210C, H393P, and L498P), individually introduced into the wild-type 4S cDNA by site-directed in vitro mutagenesis, and transfected into Chinese hamster ovary cells. Three of the five mutations (R95Q, Y210C, and H393P) were observed in >1 of 25 unrelated MPS-VI patients; however, the mutations were not found in 20 control individuals. The residual 4S activity and protein (biochemical phenotype) were determined for each mutant in order to confirm their identity as mutations and to dissect the contribution of each mutant allele to the overall clinical phenotype of the patient. For each patient, the combined biochemical phenotypes of the two 4S mutant alleles demonstrated a good correspondence with the observed clinical phenotype (with the possible exception of a patient who was a compound heterozygote for T92M and L498P). This preliminary correspondence between genotype and the phenotype in MPS-VI may, with further refinement, contribute to the assessment of therapeutic approaches for MPS-VI patients.

Introduction

Mucopolysaccharidosis type VI (MPS-VI), or Maroteaux-Lamy syndrome, is an autosomal recessive disorder caused by a deficiency of the lysosomal enzyme *N*-acetylgalactosamine-4-sulfatase (4S; EC 3.1.6.12), also known as "arylsulfatase B" (Maroteaux et al. 1963; Stumpf et al. 1973; Hopwood and Morris 1990; Neufeld and Muenzer 1995). The disease is characterized biochemically by the intralysosomal storage and urinary excretion of elevated levels of the glycosaminoglycan, dermatan sulfate. Clinical features can include abnormal growth, short stature, stiff joints, skeletal malformations, corneal clouding, hepatosplenomegaly, and cardiac abnormalities. A wide variation in clinical severity is observed between MPS-VI patients, ranging in a spectrum from relatively mild to severe forms, on the basis of age at onset, the extent of organ involvement, and the rate of disease progression. Attempts to correlate disease severity with residual 4S activity or 4S catalytic capacity in patient fibroblasts or with dermatan sulfate levels in patient urine have been partially successful (Hopwood and Elliott 1985; Brooks et al. 1991; Brooks 1993).

The cloning of the 4S gene (Peters et al. 1990; Schuchman et al. 1990; Litjens et al. 1991) has enabled the identification and analysis of 4S mutations in MPS-VI patients (Wicker et al. 1991; Jin et al. 1992; Litjens et al. 1992; Arlt et al. 1994; Isbrandt et al. 1994; Voskoboeva et al. 1994). These reports demonstrated that there is genetic heterogeneity of 4S mutant alleles, and this fact may underlie the clinical variation in MPS-VI patients. Here, we report five missense mutations identified in MPS-VI patients with varying clinical severities. In order to determine the contribution of each mutation to the clinical phenotype, the mutations were generated in vitro by oligonucleotide-directed mutagenesis, expressed in Chinese hamster ovary (CHO) cells, and the residual 4S activity and protein (biochemical phenotype) were measured. The combined biochemical phenotypes of the two mutant 4S alleles were then compared with the observed patient clinical phenotype.

Patients, Material, and Methods

Patients

The clinical phenotype of MPS-VI patients was classified as severe, intermediate, or mild, on the basis of the

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age at clinical diagnosis and the range of symptoms. The classification is relatively crude, because the clinical information was provided by different physicians with different terms of reference.

All patients except for SF693 and 51-1 were diagnosed by measurement of reduced 4S enzyme activity in leukocytes and cultured fibroblasts by use of a radiolabeled oligosaccharide substrate derived from chondroitin-4-sulfate (Hopwood et al. 1986). Patients SF693 and 51-1 were diagnosed by measurement of elevated concentrations of dermatan sulfate excreted in the urine and by measurement of reduced 4S enzyme activity in cultured fibroblasts toward the fluorogenic substrate, 4-methylumbelliferyl sulfate. Immunoquantification was used to measure residual 4S protein in patient cultured fibroblasts by using a panel of seven anti-4S monoclonal antibodies (Brooks et al. 1991). Fibroblast extracts from the patients had residual 4S activity and protein levels <5% of normal controls.

Patient SF1246 was classified clinically as a severely affected MPS-VI patient, because she originally presented at the age of 13 mo with mild developmental delay and thoracolumbar kyphosis. Hepatosplenomegaly was present, and radiological examination found skeletal features consistent with an MPS.

Patient SF693 was also classified with a severe phenotype. She presented at the age of 3 years 9 mo with coarse features, corneal clouding, and some restriction of movement in all joints.

Patient SF912 presented at the age of 13 years with short stature; she was 133.5 cm in height (<3d centile) and 30.3 kg in weight (<3d centile). The patient had a slight kyphosis, hearing loss in both ears (30 dB), mild mitral valve incompetence, a normal electrocardiogram, and no splenomegaly. Subsequently, an older brother SF913 (16 years of age) was diagnosed during the clinical investigation of the family. Patients SF912 and SF913 were classified as clinically mild, on the basis of the later age at diagnosis and the reduced extent and progression of symptoms relative to classical severe patients.

Patient 51-1 was also classified with a mild phenotype. She presented at 15 years of age because of short stature (142 cm in height [<5th centile] and weighed 45 kg [10th centile]). The patient had a normal facial appearance, enlarged liver, very slight restriction of large joints, fixed flexion deformity at terminal interphalangeal joints, and a very slight degree of corneal clouding. A soft systolic heart murmur was noted, while echocardiography was normal.

Patient SF2724 was previously diagnosed clinically (incorrectly) with Morquio syndrome at the age of 11 years. At the age of 17.5 years, the patient presented with hearing loss. He was short (10th centile), had coarse facial features, and had restricted movement in the shoulder, hip, and other joints. Corneal clouding was slight, and no hepatosplenomegaly was observed. Subsequent biochemical investigation corrected the diagnosis to MPS-VI.

Patient SF839 presented at the age of 17 years with radiological features consistent with MPS-VI. She had cloudy corneas, very mild hydrocephalus, and a very slight compression of the spinal cord.

Patient SF2984 was diagnosed at 6 years and 8 mo. She presented with corneal clouding, skeletal abnormalities, and restriction in movement of the hip and shoulder joints. Facial dysmorphism was evident by 8 years. The patient's clinical phenotype was classified as intermediate, because it lay between the mild and severe extremes.

The SF2467 cell line was obtained from the Human Genetic Mutant Cell Repository (GM2849; Camden, NJ). The patient was considered a mild MPS-VI, because he was diagnosed at 35 years of age; however, no clinical details were provided.

DNA-Template Generation and Amplification

Genomic DNA was isolated from cultured fibroblasts according to a modification of the procedure of Nelson et al. (1989). Total RNA was isolated from normal and patient cultured skin fibroblasts and used in the synthesis of first-strand cDNA as described elsewhere (Litjens et al. 1992). The entire coding region of the 4S cDNA was PCR-amplified in two overlapping portions, PCR A and B, as described by Litjens et al. (1992), except that the PCR B reaction was performed without 10% dimethylsulfoxide. The oligonucleotide primers used for further PCR amplification are listed in table 1.

Mutation Characterization

The 1.65-kb *Eco*RI-*Stu*I fragment from the modified 4S cDNA clone p4SFL (Anson et al. 1992) was used as the reference control for chemical cleavage. Labeled PCR A and B probes were synthesized from control and patient DNA and screened using a modified chemical cleavage of mismatch technique (Forrest et al. 1991; Litjens et al. 1992). The direct DNA sequencing of PCR products was performed as described by Litjens et al. (1992) by use of the oligonucleotide primers listed in table 1. The presence of each identified base change was assayed by allele-specific oligonucleotide (ASO) hybridization (Conner et al. 1983) to PCR products derived from genomic DNA samples of 20 unrelated normal control individuals and 25 unrelated MPS-VI patients, to determine allele frequencies in the two populations. The sequences of the PCR primers and ASOs are listed in table 1.

Mutant 4S Expression Constructs

The 4S cDNA template for mutagenesis was the 2.2-kb 4S cDNA *Eco*RI fragment isolated from pASB2-5 (Peters et al. 1990), which was subcloned into M13mp18 in the antisense orientation. The pASB2-5 clone encoded the complete 4S reading frame, except for the first 13 amino

Table 1

Oligonucleotides Used in the Present Study

Primer	DNA Sequence (5′→3′)[a]	Position[b]	Sense (s) or Antisense (a)
PCR/sequencing primers:			
4SP16	CGGCAGCCCAGTTCCTCATT	−79 to −60	s
4SP5A	ATGGGTCCGCGCGGCGCGGCG	1 to 21	s
4SP17	GCCGGCGGGGTGCTCCTGG	229 to 247	s
4SP14	TAGCGGCCAGTGAGCAGCTG	289 to 308	a
4SP11	TCATCCAGAGGAACACAGCT	358 to 377	a
4SP21	GGAAGGCATTCTTTCCGGTA	451 to 470	a
4SP12	TAATCTTCACTACCCAGGAG	505 to 524	a
4SP22	GAGATGGCGAAGAAGTTGCA	590 to 609	s
4SP30	TCTCTGGTGGATGGTTAGTT	669 to 688	a
4SP23	CTGCATAGTGATGCCTGTTC	783 to 802	a
4SP6	CTATGCAGGAATGGTGTCCC	795 to 814	s
4SP7	GCCAGTTATTACCCCCTGCC	918 to 937	a
4SP24	GCAGGGGGTAATAACTGGCC	919 to 938	s
4SP26	TCCTTCACTGATGGTTTTCC	1130 to 1149	a
4SP31	TGAAGGAAGCCCATCCCCCA	1143 to 1162	s
4SP32	CGGTGAAGAGTCCACGAAGT	1193 to 1232	a
4SP27	AACACATCTGTCCATGCTGC	1276 to 1295	s
4SP28	AGCCCGTGAGGAGTTTCCAA	1311 to 1330	a
4SP33	GCTGTGGTTACTGGTTCCCT	1337 to 1356	s
4SP8	TGAAAGGTTTTCTAGCCTCC	1611 to 1630	a
ASO primers:			
4SThr92	GCTGTGCACGCCGTCG	267 to 282	s
4SMet92b	CGACGGCATGCACAGC	267 to 282	a
4SArg95	CGTCGCGGAGCCAGCTG	278 to 294	s
4SGln95	CGTCGCAGAGCCAGCTG	278 to 294	s
4STyr210	TAAAAATATGTATTCAACAA	618 to 637	s
4SCys210	TAAAAATATGTGTTCAACAA	618 to 637	s
4SHis393	CTGCTGCATAATATTGACCC	1171 to 1190	s
4SPro393	CTGCTGCCTAATATTGACCC	1171 to 1190	s
4SLeu498	GTCACAAAGCTCCTGTCCC	1483 to 1501	s
4SPro498	GTCACAAAGCCCCTGTCCC	1493 to 1501	s

[a] Allele-specific nucleotides are underlined.
[b] Nucleotides numbered according to Peters et al. (1990), Schuchman et al. (1990).

acid codons (nucleotides 1–38) at the 5′ end. Site-directed in vitro mutagenesis of the 4S cDNA was performed using a commercially available mutagenesis kit (Amersham) according to the manufacturer's instructions. The kit was based on the phosphorothioate approach of Nakamaye and Eckstein (1986). To facilitate the assembly of the mutant 4S expression constructs, each of the mutations was excised from the in vitro mutagenized 4S cDNA clones as a small "cassette" fragment by restriction endonuclease digestion. The T92M and R95Q mutations were individually isolated on a 300-bp *Avr*II-*Bsm*I cassette, the Y210C mutation was isolated on a 272-bp *Dra*III-*Pst*I cassette, and the H393P and L498P mutations were individually isolated on a 549-bp *Sac*I-*Apa*I cassette. Each mutant cassette was then cloned into the appropriate pUCXB4S "cassette-acceptor" vector. These vectors contained the modified full-length 4S cDNA clone (obtained from the 4S expression vector pRSVN.4S.08 [Anson et al. 1992] by digestion with *Xba*I and *Bam*HI), from which the corresponding wild-type cassette had been removed by restriction endonuclease digestion. The pUCXB4S cassette-acceptor vectors contained a polylinker in which the standard pUC18 polylinker was removed and replaced with a truncated polylinker containing only *Hind*III, *Xba*I, *Bam*HI, and *Eco*RI sites, respectively. The mutant 4S cDNAs were excised from the pUCXB4S constructs and then directionally recloned into the *Xba*I and *Bam*HI sites of the pRSVN.4S.08 expression construct from which the wild-type 4S cDNA had been previously removed.

CHO Cell Transfection

CHO cells (strain DK1) were grown in F-12 nutrient media (Ham's), supplemented with 10%–20% (v/v) heat-inactivated (56°C for 60 min) FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin sulfate, and 120 μg/ml kanamycin sulfate at 37°C in a 5% CO_2/air atmosphere.

The cells were fed with fresh medium every 2–3 d. Cells were typically cultured in either 75-cm^2 culture flasks or 100-mm-diameter culture dishes, with 10–13 ml of nutrient media. Wild-type and mutant 4S cDNA expression vector constructs were introduced into CHO cells by electroporation, as described by Anson et al. (1992). Stably transfected cells were selected for by G418 resistance (750 μg/ml). The cultured adherent cells were harvested when confluent by trypsinization, the cells were resuspended in extraction buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 7.0), and lysates were prepared by six freeze-thaw cycles alternating between dry ice/ethanol and tepid water. The cell lysates were clarified by centrifugation at 12,000 g for 5 min at 4°C. The total protein content of the extracts was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions, using known quantities of BSA as the standard. The cell extracts were then assayed for arylsulfatase activity with the fluorogenic substrate 4-methylumbelliferyl sulfate (4MUS), and the 4S protein was quantified by enzyme-linked immunoadsorbant assay as described below.

4S Measurements

Two approaches were used to determine arylsulfatase activity toward 4MUS. In the first approach, the total arylsulfatase activity (arylsulfatases A, B, and C) of each sample was measured in a direct assay and was performed as described by Gibson et al. (1987). The second approach utilized immunocapture with various monoclonal antibodies to purify human 4S away from other CHO arylsulfatases and therefore permit the detection of 4S-specific arylsulfatase activity (Brooks et al. 1995). The monoclonal antibodies 4S 4.1, 15.1, 16.1, 33.1, 56.2, 58.3, 59.2, and F66 were as described elsewhere (Gibson et al. 1987; Brooks et al. 1991, 1995). 4S polypeptide levels were determined with an immunoquantification assay, which used an anti-4S polyclonal antibody to capture the protein and one of a panel of anti-4S monoclonal antibodies and a peroxidase-labeled second antibody to detect and quantify the bound protein (Brooks et al. 1991).

Results and Discussion

Identification of 4S Mutations in MPS-VI Patients

Southern blot analysis of genomic DNA from a panel of 17 unrelated MPS-VI patients (which included SF839, 912/913, 1246, 2467, and 2724) failed to detect major deletions or rearrangements of the 4S gene, relative to normal controls (data not shown). A subset of patient samples (SF913, SF1246, and SF2467) was then selected for chemical cleavage analysis, because analysis of the entire panel was not considered feasible with the available resources. The three patients were selected because together they were representative of the clinical severity spectrum and therefore might provide an insight into the molecular basis for the variation in clinical severity. The cleavage bands observed and the predicted mismatch sites for each patient are summarized in table 2. PCR products spanning the predicted sites of cleavage were generated and the DNA sequences were determined by direct DNA sequencing of PCR products (table 2). Five amino acid substitutions (T92M, R95Q, Y210C, H393P, and L498P) were identified in these three patients and a previously reported silent polymorphism (A or G at nucleotide position 1191; 1191G/A) (summarized in table 2).

Table 2

Mutations Identified by Chemical Cleavage

Cell Line	PCR	Fragment Size[a,b] (bp)	Predicted Nucleotide Position	Base Change[c]	Amino Acid Change
SF1246	A-7	660 H	280	G284A ht	R95Q
	B-6	380 H, OT	1180	A1178C ht	H393P
	B-8	445 OT	1185	A1178C ht	H393P
	B-8	430 H, OT	1200	1191G/A ht	...
SF913	A-7	630 H	280	G284A ht	R95Q
		310 H	630	A629G ht	Y210C
	B-8	430H	1200	1191G	...
SF2467	A-5A	270H	270	C275T ht	T92M
	B-6	700H	1495	T1493C ht	L498P
	B-8	430H	1200	1191G/A ht	...

[a] The cleaved PCR probe and the labeling primer are shown.
[b] "H" indicates fragment observed in hydroxylamine reaction; "OT" indicates fragment observed in osmium tetroxide reaction.
[c] "ht" denotes heterozygosity.

ASO hybridization was used to confirm the identified mutations and also to determine the frequency of the identified base changes in a collection of 25 unrelated MPS-VI patients and 20 unrelated normal (non-MPS-VI) individuals. Three of the mutations were found in more than one patient; Y210C and H393P were each found in four unrelated patients (SF2984, SF912/913, 51-1, SF2724) and (SF1246, SF693, SF2984, SF839), respectively, and R95Q was found in two unrelated patients (SF1246, SF912/913) (table 3). Together, R95Q, Y210C, and H393P represented 20% of mutant alleles (10/50). T92M and L498P were each unique to a single patient. All nine patients were compound heterozygotes, in contrast to the results of Isbrandt et al. (1994), who identified six different 4S mutant alleles present in six patients, all of whom were homozygotes. The number of heterogeneous mutations in the 4S gene of MPS-VI patients was consistent with the broad clinical phenotype seen in this disorder (table 3). It is interesting to note that the T92M and R95Q mutations occurred in the CTPSR peptide sequence, a region highly conserved among sulfatases (Wilson et al. 1990). In particular, the

Table 3

Summary of MPS-VI Patient Genotype and Phenotype

Patient	Mutant Allele 1	Mutant Allele 2	Urinary Dermatan Sulfate[a]	Age at Clinical Diagnosis	Clinical Phenotype
SF1246	R95Q	H393P	NA	13 mo	Severe
SF693	H393P	NI	NA	3.75 years	Severe
SF2984	Y210C	H393P	NA	6.7 years	Intermediate
SF2724	Y210C	NI	4	11 years	Mild
SF912	R95Q	Y210C	8	13 years	Mild
SF913	R95Q	Y210C	9	16 years	Mild
51-1	Y210C	NI	NA	15 years	Mild
SF839	H393P	NI	NA	17 years	Mild
SF2467	T92M	L498P	NA	35 years	Mild

NOTE.—NA = not available; NI = allele not identified. Mutant alleles in SF1246, 913, and 2467 were identified by chemical cleavage analysis and DNA sequencing. The other patients were then screened for the presence of these mutant alleles by using ASO hybridization.

[a] Urinary dermatan sulfate as reported by Brooks et al. (1991). Normal controls (>6 years) = .7 g/mol creatinine. Values for severe MPS-VI patients range from 34 to 127 g/mol creatinine (see Brooks et al. 1991).

T92M and R95Q mutations occur at positions that are conserved in four of seven and seven of seven sulfatases, respectively (Tomatsu et al. 1991).

Expression and Characterization of Mutant 4S

In compound heterozygous patients, biochemical analysis is made complex by the presence of two different mutant 4S proteins, which together contribute to the observed clinical and biochemical phenotype. In order to resolve the individual effect of each of the 4S mutations on 4S activity and protein, the mutant alleles were individually constructed by site-directed mutagenesis. The normal 4S and the five mutant expression vectors derived from the wild-type 4S cDNA expression construct pRSVN.4S.08, were assembled as described in Methods and, were designated "4S-N," "4S-T92M," "4S-R95Q," "4S-Y210C," "4S-H393P," and "4S-L498P." Prior to transfection into CHO cells, the DNA sequence of each of the 4S mutations was confirmed by ASO hybridization analysis. Stably transfected clonal lines were isolated by G418 selection (5–12 clones per construct), and the total arylsulfatase activity was determined in the cell extract and the culture medium. The two clonal cell lines with the highest activity for each 4S construct were selected for further analysis. The total arylsulfatase activity, immunocaptured 4S activity, and the 4S protein were determined on cellular extracts.

The total arylsulfatase and 4S activities of the cell extracts from the 4S mutant cell lines were all markedly reduced relative to the wild-type human 4S control (table 4). The residual activity and protein values were similar for the two clonal lines of each 4S mutant. The Y210C mutants had the highest residual total arylsulfatase activity of the 4S mutants (~2% of wild-type total arylsulfatase activity), while the T92M mutants had a very low residual activity (~0.03% of wild-type). The residual activity of the R95Q mutants was ~0.02% and thus at the resolution limit of the total arylsulfatase assay to distinguish above background activity. The total arylsulfatase activities of the H393P and L498P mutants were indistinguishable from the CHO control. An immunocapture assay was used to determine the level of specific 4S activity in the mutant cell lines and gave similar results to the level of arylsulfatase activity (table 4). Moreover, immunocapture using the monoclonal antibodies 4S 4.1, 4S 56.2, 4S 58.3, and 4S F66 demonstrated similar levels of activity for each antibody on the same cell line (data not shown). This indicated that the reduced 4S activity in the immunocapture assay was not due to a single missing epitope.

The 4S protein from the cell extracts of the 4S mutants were all markedly reduced (<5%) relative to the wild-type control (table 4). In particular, the H393P and L498P mutants had no immunodetectable 4S protein. The reduced concentration of mutant 4S proteins relative to the wild-type control was unlikely to be an assay

Table 4

Biochemical Measurements of Expressed 4S Mutants

Cell Extract	Total ARS Activity[a] (units/mg)[b]	4S-Specific ARS Activity[a,c] (units/mg)	4S Protein[d] (μg/mg)	Specific Activity[e] (units/μg 4S)
4S-N.3	560	1,080	57	19
4S-N.3	620	1,044	56	19
4S-N.MC	300	552	28	20
4S-T92M.4[f]	.18	.1–.4	.13	.7–2.8
4S-T92M.9[f]	.21	.1–.4	.17	.5–2.7
4S-R95Q.4[f]	.10	.03	.88	.03
4S-R95Q.12[f]	.11	.03	.76	.03
4S-Y210C.2	12	14	2.3	6.1
4S-Y210C.5	14	18	2.8	6.6
4S-H393P.2	.05	.02	ND	...
4S-H393P.11	.06	.01	ND	...
4S-L498P.1	.05	.01	ND	...
4S-L498P.7	.05	.02	ND	...
CHO control	.05	.01	ND	...
CHO control	.04	.01	ND	...

NOTE.—ARS = arylsulfatase; MC = mass culture; ND = not detected.

[a] Activity measured using 4MUS.

[b] Units (nmol/min)/mg of cell extract protein.

[c] Immunocaptured 4S-specific activity using ASB F66.

[d] 4S protein (μg/mg of cell extract protein) was immunoquantified using ASB F66.

[e] Specific activity (nmol/min/μg 4S protein), corrected for CHO background.

[f] The activities of the T92M and R95Q mutants in the cell extracts were concentration dependent.

artifact due to a reduced affinity of 4S F66 for the mutant proteins, since the immunocapture assay demonstrated similar levels of activity as the arylsulfatase assay for each cell line. No significant 4S-specific activity was found in the culture medium for any of the 4S mutants, which ruled out incorrect targeting to the cell surface via the secretory pathway as an explanation for the low intracellular activity and protein of the 4S mutants.

Comparison of the Biochemical and Clinical Phenotype

In cultured MPS-VI patient fibroblasts the entire spectrum of clinical phenotypes from severe to mild is clustered over a low and narrow range of residual activity from 0% to <5% of normal controls (Brooks et al. 1991). Conzelmann and Sandhoff (1983/84) have proposed a model to account for the observation that a number of lysosomal storage disorders have low residual activity of mutant lysosomal proteins. The model predicts that low residual activities, down to a critical threshold level, may be sufficient to metabolize substrate and avert lysosomal storage. Below the threshold value, lysosomal storage will occur such that small changes in residual activity may lead to large differences in the rate of substrate storage and result in pronounced differences in clinical severity. The considerably reduced level of dermatan sulfate found in the urine of clinically mild patients (SF2724, SF912, SF913) compared with the level in clinically severe patients (table 3) was consistent with the proposal that the level of storage reflects the severity of the clinical phenotype. Thus, the H393P and L498P mutations would be associated with the most severe clinical phenotypes, because of the complete absence of detectable protein and activity, whereas R95Q, T92M, and Y210C should result in progressively less severe MPS-VI clinical phenotypes, because of the increasing levels of 4S activity found in these mutants.

In compound heterozygous patients, it was anticipated that the Y210C allele would reduce the severity of more severe alleles, because of its relatively high residual activity. This prediction was supported by the later age at diagnosis of the five patients who were heterozygous for the Y210C mutation (table 3). Although patients heterozygous for the Y210C allele were generally at the milder end of the clinical spectrum, there was still some variation in severity. Confirmation that both the R95Q and H393P alleles were associated with a severe clinical phenotype came from the observation of a severely affected patient (SF1246) who was heterozygous for both alleles. The biochemical phenotypes of the T92M and L498P alleles were difficult to reconcile with the clinical severity of patient SF2467. The phenotype of a T92M/L498P compound heterozygote should be determined by the residual activity associated with the T92M allele, since L498P had essentially no activity. The T92M allele expressed at least fourfold greater residual activity than R95Q but less than one tenth that expressed by Y210C. Therefore, patient SF2467 was expected to have a clinical presentation somewhere between that of the severe patient SF1246, who is a compound heterozygote of R95Q and the null allele H393P (on the basis of the absence of residual 4S activity or protein), and the mild patients who carry the Y210C mutation. However, according to the information provided by the cell repository from which the patient cell line was obtained, the patient was not diagnosed until the late age of 35 years, which suggests a mild phenotype. Unfortunately, the actual clinical severity of this patient was not known, as no clinical details apart from the age at diagnosis were provided. In addition, the T92M and L498P alleles were unique to SF2467 in the patient collection screened, and therefore the discrepancy between the biochemical phenotype of each allele and the apparent clinical phenotype of the patient could not be resolved by comparison with the phenotypes of other patients.

It is clear that the determination of a mutation's biochemical phenotype in a heterologous high-level expression system using an artificial substrate does not necessarily reflect the molecular events within the lysosome and should only be used as a guide to predict phenotype. An example of variation in vitro that complicated determination of the biochemical phenotype was the dilution effect observed with both T92M and R95Q. T92M had residual activity upon dilution of $\leq$2.0 units/mg of cell extract protein, compared to 0.1 units/mg in neat samples, while R95Q had residual activity upon dilution of $\leq$1.2 units/mg, compared to 0.03 units/mg in neat samples (data not shown). T92M also showed a different pH optimum, with threefold greater activity at pH 4.6 than at the normal optimum of pH 5.3 (data not shown).

In summary, the 4S mutant alleles Y210C, R95Q, and H393P showed a good correspondence with the patient clinical phenotype, in light of the inherent limitations in the clinical information available and the biochemical assay. The correspondence was strengthened for each of these mutant alleles because each was observed in more than one patient. However, the biochemical phenotype of the T92M and L498P alleles was somewhat discordant with the reported clinical severity of the single patient in whom these alleles were found.

The tentative association between genotype and phenotype in MPS-VI reported in this study may, with further refinement, contribute to the prediction of disease progression and the assessment of various therapeutic approaches in MPS-VI patients. Improved understanding of genotype-phenotype correspondence in MPS-VI may be obtained with a larger patient base, a comprehensive and standardized protocol for assessment of clinical severity, and a more complete analysis of the effect of other intragenic sequence variations on the expression of residual 4S activity. Analysis of patients who

do not fit the observed pattern of genotype-phenotype correspondence may reveal deeper insights into the molecular mechanisms and/or environmental factors that modulate disease severity.

Molecular Mechanisms of 4S Deficiency

The 4S mutations characterized in this study all led to reductions in 4S activity and protein to <5% of wild type. The magnitude of these reductions were similar to those observed in patient cultured cells (Brooks et al. 1991; Brooks 1993). The reduction in the total amount of 4S protein appears to be the major contributor to the deficiency of 4S activity in MPS-VI patients. A number of studies have proposed that mutant polypeptides are retained and degraded in a compartment in or near the endoplasmic reticulum (ER) (Lodish 1988; Lippincott-Schwartz et al. 1988). It is conceivable that the mutant 4S proteins may be recognized as aberrant and degraded during the processes of translocation and folding of the nascent polypeptide by ER-resident molecular chaperones (Arlt et al. 1994; Brooks et al. 1995). Another possibility is that the mutant 4S may enter the ER but be missorted to a nonlysosomal destination, where it cannot fulfil its physiological role. Yet another possibility is that the mutant 4S enzymes may also conceivably be correctly targeted to the lysosome but exhibit markedly reduced stability in the hydrolytic environment. Whatever the mechanisms responsible for the reduced 4S protein, they exhibit a high degree of sensitivity and are particularly stringent, since the levels of all five mutant 4S proteins were markedly reduced. In addition to protein reduction, a mutation may mediate its effects through reduction in the specific activity of the enzyme toward its substrate. Of particular interest was the R95Q mutation, which was a nonconservative change of a residue totally conserved between seven sulfatases (Tomatsu et al. 1991). We observed a marked, but not total, reduction in specific activity of 4-sulfatase as a result of this mutation. It is conceivable that the R95Q mutation may directly perturb critical residues involved in catalysis and/or substrate binding and/or residues that are crucial to the molecular architecture of the enzyme. Mutation of the conserved amino acid C91, in the CTPSR region of 4-sulfatase, was shown to completely abrogate 4-sulfatase activity (Brooks et al. 1995). Further, Schmidt et al. (1995) have proposed that the modification of this C91 residue to a 2-amino-3-oxopropionic acid residue is necessary for the generation of catalytically active sulfatases. The R95Q mutation may therefore cause a structural modification in 4-sulfatase, which prevents the correct processing of this C91 residue.

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